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Letter to the Editor

Lecithin:retinol acyltransferase in ARPE-19

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Abstract

The purpose of this study is to investigate if a readily available cell line (APRE 19) may be used to study in vitro function of visual cycle enzymes such as lecithin:retinol acyltransferase (LRAT). Cells incubated with exogenous retinol accumulated intracellular all *trans* retinol and all *trans* retinyl ester. Membrane proteins from ARPE 19 exhibited LRAT activity, which was inhibited by an LRAT inhibitor, retinyl bromoacetate (RBA). Gene microarray and Western blot results indicated that ARPE 19 cells expressed LRAT transcript and the LRAT protein. Therefore, our data show that ARPE 19 contains an active LRAT enzyme and suggest that it is an appropriate cell system to study visual cycle enzymes.

Keywords: LRAT; retinal pigment epithelium; vitamin A; retinyl ester; ARPE 19

In the vertebrate retinal pigment epithelium (RPE), all-*trans* retinol is processed into 11-*cis* retinal to regenerate visual pigments. In the RPE microsomal membrane, all-*trans* retinol is esterified by LRAT (Saari and Bredberg, 1988). Acyl-CoA:retinol acyltransferase (ARAT) also contributes to retinyl ester formation (Saari and Bredberg, 1989, 1990). Recently, a truncated form of LRAT (tLRAT) has been extensively studied (Bok et al., 2003) leading to the development of tLRAT antibody (Dr. Dean Bok, UCLA). All-*trans* retinyl esters are isomerized to 11-*cis* retinol by isomerohydrolase (Moiseyev et al., 2003; Gollapalli and Rando, 2003). This isomerization is highly dependent on cellular retinal binding protein (CRALBP) (Winston and Rando, 1998; Stecher et al., 1999) and RPE65 (Redmond et al., 1998). 11-*cis* retinol is then oxidized by a retinol dehydrogenase to 11-*cis* retinal to form rod and cone pigments.

Subcellular organizations and protein protein interactions are of great importance in the proper processing of retinoids in the visual cycle (for a summary, see Lamb and Pugh, 2004). Therefore, it is appropriate to study the function of visual cycle enzymes in an intact cell system, rather than in buffered solutions. ARPE-19, a non-transformed adult human RPE cell line, retains many of the morphological features of RPE and expresses RPE65 mRNA and CRALBP (Dunn et al., 1996). Surprisingly, investigations of visual cycle enzymes, such as LRAT, have not been reported in these cells. In the present study, we have conducted enzyme assays and protein expression studies to show the presence of an active LRAT enzyme in ARPE-19 cells. The demonstration of in vitro LRAT activity in such a whole cell system suggests that this cell line offers an excellent opportunity to study visual cycle enzymes in their native intracellular environment.

ARPE-19 cells were purchased at P21 from ATCC, grown to P27 and stored in liquid nitrogen. Revived cells were grown to confluency according to supplier's instructions. For gene microarray studies, cells were cultured as previously described (Dunn et al., 1996). Membrane proteins from ARPE-19 were collected from cell homogenate after centrifugation at 100 000 g for 30 min at 4°C. Microsomal protein from bovine RPE was prepared by previously described methods (Saari and Bredberg, 1988).

Cells grown to confluency were incubated with a 10 μM all-*trans* retinol (Sigma), 500 μg ml ^{1} fatty acid free bovine serum albumin (BSA), and 0.5% sucrose solution. Cells were washed, harvested and homogenized in phosphate buffered

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solution, pH 7.0. Retinoids were extracted by methanol and dichloromethane and analysed by HPLC (Waters HPLC with autosampler and photodiode array detector) on a dioxane/-hexane gradient. Retinoids were identified based on relative retention, coeultion with standards and online photodiode array spectrum and quantified against authentic standard curves (Waters Millenium Software). The identity of retinyl esters was confirmed by saponification in strong base (10.6 M KOH ethanolic solution) with the retinol products analysed by HPLC.

LRAT activity assays were carried out as described by Saari and Bredberg (1989). In our study, 20 μM all-*trans* retinol was preincubated with BSA (60 μM fatty acid free) before the addition of membrane protein. To study ester synthesis attributable to ARAT, 20 μM palmitoyl-CoA (Sigma) was incubated with membrane proteins for 10 min prior to the introduction of retinol. For LRAT inhibition studies, RBA was synthesized as previously described (Mata et al., 1992). Membrane proteins were incubated without and with RBA (10 μM) 5 min prior to the addition of substrate. All enzyme assays were carried out in triplicate.

Isolated, total RNA was assessed spectrophotometrically and by agarose gel electrophoresis. cDNA slides were created with a GeneMachine (Genomic Solutions) robot, which spotted 70 mer oligimers for 21 000 genes on epoxy-coated slides. Control cDNA was labeled with a CY3 fluorescent probe (Amersham Biosciences), cleaned on QaiQuick Spin columns (Qiagen Inc.), and hybridized for 20 hr in a 40°C oven within an AneroPack chamber humidified with dH2O. Slides were scanned with a GenePix 4000B scanner (Axon Instruments, Inc.) and data acquired and analysed with GenePix Pro 4.1 software. Gene Pix result files were used and median fluoresecent intensities at 532 nm minus the background generated control sample values. Fluorescence intensities from five controls were normalized to reflect equal RNA amounts from each sample. Means and standard error of the means were determined.

Membrane proteins from bovine RPE, ARPE-19 (harvested at and one week beyond confluency), and chicken glial cells (devoid of LRAT activity), were separated on a SDS-PAGE 15% acrylamide gel, in denaturing conditions. Proteins were transferred to a PVDF sheet (Amersham) for the detection of tLRAT (gift from Dr. Dean Bok, UCLA) by Western blot. The blot was treated with biotinylated secondary antibody and then the avidin horseradish peroxidase complex (Vectastain). Bands were visualized with Vectastain DAB horseradish peroxidase substrate kit according to manufacturer's instruction.

To study the esterification of exogenous all-*trans* retinol by ARPE-19, we incubated cells with all-*trans* retinol (10 μM) and determined retinyl ester formation over time. There was a rapid accumulation of intracellular all-*trans* retinol (~15 pmol μg ^{1} protein) within the 24-hr period (Fig. 1A). This reflects our assay condition in which the retinol substrate was not removed from the media throughout the incubation. In contrast, there was a gradual

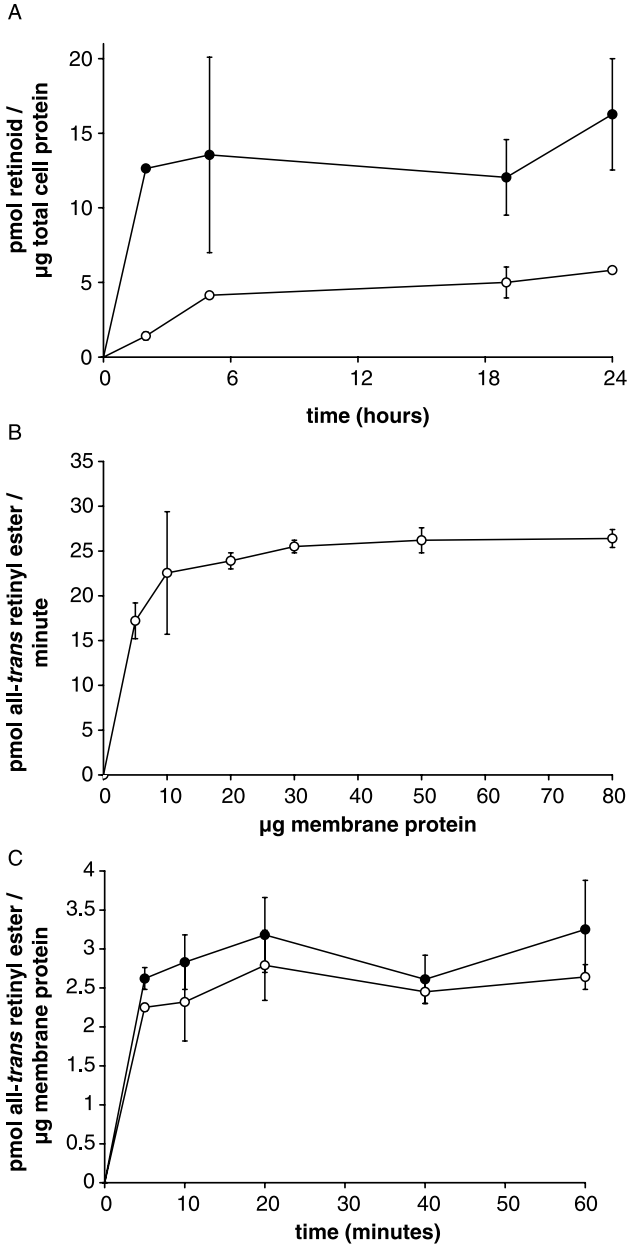

Fig. 1. LRAT mediated esterification of retinol by ARPE 19 in culture and by membrane protein. (A) Time dependent accumulation of all *trans* retinol (*closed circles*) and all *trans* retinyl ester (*open circles*) in ARPE 19 cells incubated with 10 μM all *trans* retinol. Results from duplicate experiments with standard error of the mean are shown. (B) Protein dependent esterification of retinol by ARPE 19 membrane protein. The reaction was terminated after 5 min of incubation with substrate. (C) Time dependent esterification of retinol by ARPE 19 membrane protein (50 μg) in the absence (*closed circles*) and presence (*open circles*) of palmitoyl CoA. Results from triplicate experiments with standard error of the mean are shown.

formation of retinyl esters which peaked at ~6.0 pmol μg ^{1} protein at the end of 24 hr. Retinyl esters eluted from HPLC were collected and saponified to yield mainly all-*trans* retinol and a small amount of 13-*cis* retinol but no 11-*cis* retinol. The identities of the fatty acids were not determined. Similar experiments carried out with radiolabeled retinol confirmed

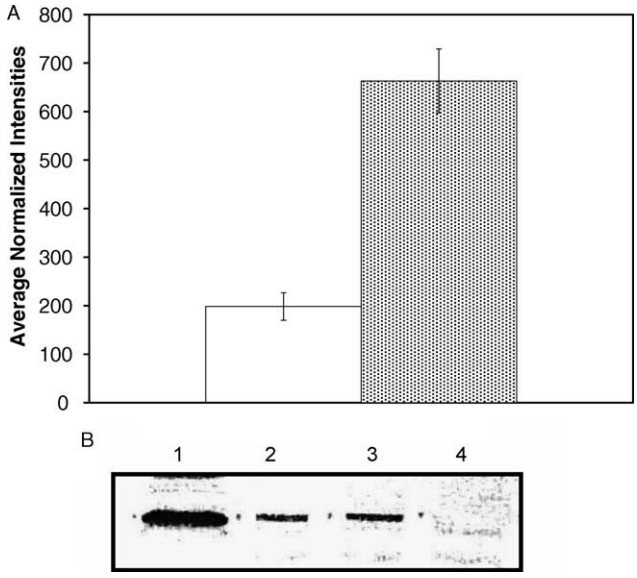

Fig. 2. LRAT microarray and Western blot analyses. (A) Microarray analysis was performed on RNA extracted from ARPE 19. Both LRAT (*white*), and housekeeping gene G3PDH (*shaded*) were detected. Normal ized intensities are shown. (B) Membrane protein (120 μg), from bovine RPE, *lane 1*; ARPE 19 at confluency, *lane 2;* ARPE 19 one week beyond confluency, *lane 3;* chicken glial cells devoid of LRAT activity, *lane 4*; were blotted with anti tLRAT antibody. The antibody recognized a band corresponding to approximately 25 kD based on calibration standards. Analysis was replicated six times.

that the intracellular retinyl esters were derived from exogenous retinol (unpublished observations). In the absence of exogenous retinol, ARPE-19 did not contain any detectable levels of retinoids.

Fig. 1B shows that the esterification of all-*trans* retinol by ARPE-19 protein is dependent on the amount of protein up to 20 μg. The addition of palmitoyl-CoA to the incubation mixture did not increase the level of esterification suggesting that there was no ARAT activity (Fig. 1C). In contrast, bovine microsomal membrane exhibited both LRAT and ARAT activity similar to the results reported previously by Saari and Bredberg (1988, data not shown). Substrate saturation analysis using ARPE-19 membrane protein yielded apparent kinetic constants (K_m and V_{max}) for all-*trans* LRAT activity of 9.5 μM and 0.84 nmol min ^{1} mg ^{1} protein, respectively. LRAT assays were also carried out in the presence of RBA, a known LRAT inhibitor (Trehan et al., 1990). In comparison to control, RBA significantly (78 ± 10%) reduced LRAT activity in ARPE-19 proteins.

Microarray analyses revealed LRAT gene is expressed by ARPE-19 cells (Fig. 2A). Expression analysis indicated that the mean fluorescence intensity level of LRAT (198.4 ± 28.3) was about one third of the house keeping gene glyceraldehydes-3-phosphate dehydrogenase (G3PDH) 663 ± 66. Western analyses with tLRAT antibody showed that LRAT protein was present in bovine RPE (positive control), ARPE-19 at confluency and one week after confluency, but not chicken glial cells lacking LRAT activity (negative control), at a molecular weight of approximately 25 kD (Fig. 2B). Based on relative band intensities, there is no significant difference between the amount of LRAT protein in ARPE-19 cells at confluency and those one week after confluency.

The identification of cell culture systems that have the capacity to internalize and process retinoids of the visual cycle has been the topic of several recent investigations (Chen et al., 2003; Cia et al., 2004). A transformed human RPE model showed both CRALBP and retinol dehydrogenase activity but no LRAT activity (Davis et al., 1995). Since RPE65 mRNA and CRALBP are expressed in ARPE-19 (Dunn et al., 1996), we wished to evaluate the presence of LRAT, a key visual cycle enzyme, in this cell system. This is the first study to report the internalization and esterification of retinol in ARPE-19. In addition, we have also conducted experiments to identify and characterize LRAT activity in ARPE-19 membrane protein. Since LRAT protein is expressed in ARPE-19 and its activity is partially inhibited by RBA, we conclude that this esterification of retinol is primarily due to LRAT. Furthermore, since this activity is not enhanced by the addition of CoA, this cell system offers a new opportunity to study the esterification of retinol by LRAT in the absence of ARAT activity.

LRAT activity in ARPE-19 membranes has an apparent K_m of 9.5 μM. This is about 50 fold higher than that reported for LRAT in bovine microsomes (0.2 μM, Shi et al., 1993). It is possible that the additional ARAT activity in bovine RPE microsomes may account for an increase of substrate affinity. It is also possible that total membranes (from ARPE-19 cells) versus microsomal proteins (from bovine RPE) may lead to differences in enzyme-substrate interactions.

In our experiments, no 11-*cis* retinol or 11-*cis* retinyl esters were detected. This suggests that isomerohydrolase may not be functional in the cell line. Flood et al. (1983) reported that 11-*cis* retinyl esters were present at primary human RPE culture but not at cells in later passages. Thus, it is possible that ARPE-19 cells of earlier passages (lower than P27) may retain isomerohydrolase activity, which remains to be studied.

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